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Toxicokinetics of atmospheric 2-nitropropane (2-NP) were investigated in rats and rabbits of both sexes. A thermodynamic partition coefficient (body/air) of 170-180 was obtained in both sexes and species from the data obtained *in vivo*. A similar value of 161 was calculated from the *in-vitro* accumulation of atmospheric 2-NP in water and olive oil. Due to metabolism in the animals, the actual concentration at steady state (body/air) was less than the former values, declining to 22-30 at concentrations below 10 ppm. 2-NP was found to be metabolized via two pathways, a non-saturable one according to first-order kinetics, which was quite similar in both sexes and species, and a saturable one according to Michealis-Menten kinetics. In rabbits no sex difference was found, and the capacity per kg body weight was similar to that of female rats. The amount metabolized by the non-saturable pathway was higher in male than in female rats and exceeded the share of the saturable pathway in males already above 2-NP concentrations of 60 ppm, in females above 180 ppm. In rabbits the respective value was reached only at 300 ppm.
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Continuation of 19. ABSTRACT

The 'rat liver foci bioassay' was performed to investigate the concentration dependence of the carcinogenic potential of 2-NP. Exposure of suckling and adult rats of both sexes to 2-NP (0-125 ppm and 0-150 ppm, respectively) for 3 weeks resulted in the formation of ATPase-deficient preneoplastic foci in the liver. Suckling females showed a higher sensitivity, but the slope of the foci incidence was equal in both sexes. In adult males, however, the foci incidence and the slope was higher than in females.

I.p. administration of 2-NP (1.7 mmol/kg b.w.) to rats resulted in an acute hepatotoxicity indicated by an increase of liver enzymes (GOT, GPT and OCT) in serum with a peak concentration after 8 h. The values in males were much higher than in females. In males, a dose-response curve was obtained with doses of 0.13 - 3.4 mmol/kg 2-NP showing a maximum after a dose of 1.7 mmol/kg.

Both, the carcinogenic and the toxic effects of 2-NP in livers of rats could be related to the non-saturable metabolic pathway. The rate constant of this pathway was very similar to that of the conversion of 2-NP to its nitronate anion. It is suggested that this metabolite leads to the hepatocarcinogenicity and hepatotoxicity of 2-NP. The lack of carcinogenic and toxic effects of 2-NP in rabbits can be explained by the low share of the non-saturable pathway up to 200 ppm.

A program "SOLVEKIN" was developed for to solve special toxicokinetic problems as simulation and parameter estimation. The program will be used for conventional compartmental and for physiologically based pharmacokinetic modelling.

This report has been reviewed and is releasable to the National Technical Information Service (NTIS). At NTIS is will be releasable to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

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FIRST DRAFT

TOXICOKINETICS, METABOLISM, AND GENOTOXICITY OF NITROPROPANES IN RATS AND MICE

Grant number: AFOSR-88-0299

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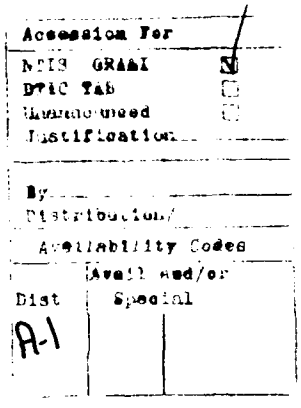

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CONTENTS

1. INTRODUCTION

2-Nitropropane (2-NP) is a widely used volatile industrial solvent. It was mutagenic in *Salmonella typhimurium* (Hite and Skeggs 1979, Löfroth et al. 1981, Speck et al. 1982, Göggelmann et al. 1988), weakly clastogenic in human lymphocytes (Bauchinger et al 1987) and genotoxic in mammalian systems *in vitro* and *in vivo* (Andrae et al. 1988, George et al. 1989, Roscher et al. 1990, Haas-Jobelius et al. 1991). In inhalation studies with Sprague-Dawley rats 2-NP was hepatotoxic and hepatocarcinogenic, male rats being more sensitive than females. Inhalation exposure of rats to concentrations of 200 ppm (7 h/d, 5 d/w, 6 month) caused liver carcinomas in both, male and female rats (Griffin et al. 1978; Lewis et al. 1979), but serum glutamic-pyruvic transaminase, a marker for hepatic injury, was elevated in exposed male rats only (Griffin et al. 1978). At an exposure level of 100 ppm hepatocellular carcinomas in males occured after 12 month of exposure, in females after 18 month only (Griffin et al. 1980). However, at the low exposure level of about 25 ppm no adverse effects of 2-NP have been observed in both sexes (Lewis et al. 1979; Griffin et al. 1980, 1981). In an inhalation study with male New Zealand White rabbits 2-NP at 25 and 200 ppm (7 h/d, 5d/w, 6 month) did not show any adverse effects (Lewis et al. 1979).

The structural isomer of 2-NP, 1-nitropropane (1-NP), was less hepatotoxic (Dayal et al. 1989) and did not show genotoxicity in *Salmonella typhimurium* (Hite and Skeggs 1979, Göggelmann et al. 1988). It was, however, found to be mutagenic in the V79/HGPRT assay (Roscher et al. 1990) and increased UDS in rat hepatocytes *in vitro* (George et al. 1989). When tested under the same conditions as 2-NP, 1-NP was not carcinogenic in rats after oral administration (Fiala et al. 1987a).

Aim of the study:

We focused our study on the most significant effects of 2-NP, namely its carcinogenic and hepatotoxic potential. As mentioned above, carcinogenicity of gaseous 2-NP has been tested in inhalation studies with male and female rats and rabbits (Griffin et al. 1978, 1980, 1981; Lewis et al. 1979).

Investigations were performed to evaluate if the sex and species differences as well as the dose-response relationships seen in these studies with rats and rabbits might be explained by sex and species specific toxicokinetics of 2-NP. As already mentioned in the Interim Report, investigations with mice were cancelled, since this species have not been used in such studies. Instead, besides male and female Sprague-Dawley rats, male and female New Zealand White rabbits were used to determine

inhalation kinetics of 2-NP. Then, a species extrapolation to man was carried out.
On the basis of the obtained toxicokinetic data the concentration-dependent carcinogenic potency of 2-NP was evaluated in male and female rats by the rat liver foci bioassay, a short-term carcinogenicity test (Oesterle and Deml 1983, 1990). To determine the age dependence, this bioassay was performed in suckling and adult animals.
The sex-specific hepatotoxicity of 2-NP after i.p. administration to SD rats was determined by measurement of the liver enzymes glutamic-oxalacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT) and ornithine carbamyl-transferase (OCT) in the blood serum (Baumann and Berauer 1985).

During the course of our study parallel investigations with the nitropropanes have been started in other working groups within the GSF. These studies included:
- Mutagenicity of 1-NP and 2-NP in S.typhimurium (W. Göggelmann, Inst. f. Toxikologie)
- Genotoxicity of 1-NP and 2-NP in rat hepatocytes *in vitro* and *in vivo* and in mammalian cell lines (U. Andrae, Inst. f. Toxikologie)
- Cytogeneticity of 1-NP and 2-NP in human lymphocytes (M. Bauchinger, Inst. f. Strahlenbiologie)
- Cytogenicity of 1-NP and 2-NP in somatic mouse cells *in vivo* (U. Kliesch, Inst. f. Säugetiergenetik)
- Mutagenicity of 2-NP in mice using the mammalian spot test and the dominant lethal test (A. Neuhäuser-Klaus, Inst. f. Säugetiergenetik)
- Teratogenicity of 2-NP (W. Schmahl, Inst. f. Pathologie)
- Metabolism of 1-NP and 2-NP in rats and primates, including the measurement of acetone exhalation and determination of GSH and lipid peroxidation in the liver (M. Haas-Jobelius, Inst. f. Ökologische Chemie)

In the light of these investigations we skipped the planned experminents with 1-NP, the metabolic investigations concerning formation of acetone and nitrite and the measurement of GSH and lipid peroxidation in the liver. The published results of these investigations, in so far as they are relevant to the subjects of our own study, have been taken into consideration (see above and DISCUSSION).

2. MATERIALS AND METHODS

2.1. Chemicals

2-NP used for kinetic studies had a purity of 95 % and was purchased from Aldrich Chemie (Steinheim, FRG). 2-NP used in the bioassays had a purity of >99 % and was a gift of Angus Chemie (Ibbenbüren-Uffeln, FRG). Clophen A50, a mixture of polychlorinated biphenyls, was obtained from Bayer (Leverkusen, FRG). All enzymes and coenzymes were from Boehringer (Mannheim, FRG). Other commercially available chemicals of analytical-reagent grade were purchased from Merck (Darmstadt, FRG) or Boehringer (Mannheim, FRG).

2.2. Animals

Male and female Sprague-Dawley rats, 200-250 g, (GSF, Neuherberg) and male and female New Zealand White rabbits, 2,3-3,5 kg, (SAVO GmbH, Kisslegg) were used for inhalation kinetics.

Rat liver foci bioassays were done with 4-6 days old male and female suckling Sprague-Dawley rats together with their dams and with adult male and female Sprague-Dawley rats, 130-190 g (GSF, Neuherberg).

2.3. Gas chromatography

Determination of 2-NP in the gas phase was carried out on a GC-8A gas chromatograph (Shimadzu, Duisburg, FRG) equipped with a 1 ml injection loop and a flame ionisation detector. Separation was done on a stainless steel column of (1/8" i.d., 2.5 m) filled with Tenax GC 60-80 mesh (Serva, Heidelberg, FRG). The oven temperature was 180°C, the gas flows were: H_2, 50 ml/min; air, 456 ml/min; N_2, 54 ml/min. Retention time of 2-NP was 1.7 min. Peak areas were determined by means of a 4290 integrator (Varian, Darmstadt, FRG).

2.4. Exposure of animals in closed chambers

Toxicokinetic parameters of 2-NP were obtained by means of the closed chamber technique (CCT, Filser 1992). Exposures of Sprague-Dawley rats or New Zealand White rabbits to initial atmospheric concentrations of100 - 3000 ppm, (rats) and of 1000 - 6000 ppm (rabbits) were performed in closed glass chambers of 19 l, occupied by 2 rats each, or of 65 l, occupied by one rabbit each. CO_2-adsorbent soda lime was omitted due to its extensive reaction with 2-NP. Every 2 h, animals were quickly transferred

into an empty chamber and the former concentration of 2-NP in the gas phase was adjusted.
Concentration-time courses in the atmosphere of the closed chambers were determined by gas chromatography.
In order to inhibit metabolism due to endoplasmic cytochrome P450 rats were pretreated 15 min before exposure by i.p. administration of dithiocarb (200 mg/kg). Rabbits were pretreated 30 minutes before exposure by i.p. administration of pyrazole (320 mg/kg).

2.5. Determination of partition coefficients

Ostwald's partition coefficients olive oil/air and water/air were determined at 37°C according to Hallier et al. (1981). Using these data the thermodynamic partition coefficient body/air was estimated as described by Filser and Bolt (1984), assuming the body of rats and rabbits to be composed of 70 % aqueous and 10 % fatty compartments (Geyer et al. 1990).

2.6. Kinetic calculations

The concentration-time courses in the atmosphere of the closed chambers were analyzed toxicokinetically by means of the two-compartment model shown in **Fig. 1**. In this model the atmosphere of the closed chamber is considered to be the first and the whole body of the exposed animals the second compartment. The processes of inhalative uptake and of exhalative elimination, determined by the clearances, $k_{12}V_1$ and $k_{21}V_2$, are treated linearily, i.e., the rates of inhalation and exhalation are directly proportional to the actual concentration in the atmosphere and to the actual average concentration in the organism, respectively. The elimination process, determined by $k_{el}{}^{*}V_2$, could be described as a function of the concentration in the organism and was composed of two different metabolic processes: a non-saturable one following first-order kinetics and a saturable one according to Michaelis-Menten kinetics. This was true for both species and sexes. Using the two-compartment model, concentration-time curves were iteratively calculated with a personal computer and fitted through the measured values. With the obtained parameters a conversion for an open system with an infinitely large atmosphere was carried out.

Species extrapolations were done assuming the apparent Michaelis-Menten constant K_{mapp} to have the same value in all species and the maximum rates of metabolism V_{max} to be proportional to the body weight. The rates of inhalation and exhalation were regarded to be limited by the alveolar

surface. Physiological processes depending on surfaces can be extrapolated between animals with different body weights using the factor (body weight)$^{2/3}$ (Filser 1992). This surface factor was calculated to be 4.64 between a rat and a rabbit and 40 between a rat and man; the respective body weight factor were 10 and 280.

2.7. Rat liver foci bioassay

Experiments were performed according to the schedule given in **Fig. 2**. Rats of two different ages were used. 4-6 days old male and female rats together with their dams or adult male and female rats (130-160 g) were kept for 3 weeks in open inhalation chambers (240 l, flow of prefiltered air of 25°C was 80 l/min). During this period animals were exposed (6 h/d, 5 d/w) to 2-NP as an initiating agent at constant concentrations (x±10%) of 0, 24, 40, 50, 80, 123 ppm (4-6 old rats) and of 0, 25, 50, 60, 125, and 150 ppm (adult rats). Liquid 2-NP was administered continuously in the air stream by an infusion pump. Atmospheric concentrations of the resulting 2-NP vapor in the air stream were controlled at the inlet and the outlet of the chamber by gas chromatography. After this initiation period animals were kept on basal diet for one week. Then, a mixture of polychlorinated biphenyls (Clophen A50, 10 mg/kg) as promoting agent was applied to all animals twice a week for 8 consecutive weeks. One week later all animals were killed under ether anesthesia. From each animal, two liver lobes were removed and frozen. Cryostat sections of livers were prepared and stained for the demonstration of foci deficient in adenosine-5'-triphosphatase (ATPase). All technical procedures were performed as described by Oesterle and Deml (1983).
Statistical analysis was done using Student's t-test for unpaired groups.

2.8. Determination of serum enzymes

Glutamic-oxalacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT) and ornithine carbamyl-transferase (OCT) as indicators of hepatotoxicity were determined in serum of rats according to Baumann and Berauer (1985).

3. RESULTS

3.1. Accumulation of atmospheric 2-NP

Enrichment of 2-NP was determined in water and olive oil. The resulting Ostwald's partition coefficients of 2-NP were 128 for water/air and 710 for olive oil/air at 37°C. The thermodynamic partition coefficient body/air (K_{eq}) for rats and rabbits was estimated from the Ostwald's partition coefficients to be 161, assuming that the body of rats and rabbits consists of 70 % aqueous and 10 % fatty compartments. A similar Keq value of 180 was obtained for rats and of 170 for rabbits from the toxicokinetic analysis of the concentration-time courses of 2-NP in the atmosphere of the exposure chamber (**Figs. 3a and b; 4a and b; Tables 1 and 2**). However, because the total metabolism of 2-NP can not be saturated (see below), the accumulation of 2-NP cannot reach this theoretical value. The ratio of the average concentration in the organism to the atmosphere at steady state (K_{st}) gives the accumulation at defined constant atmospheric concentrations. This ratio is a function of the concentration in the atmosphere (**Fig. 5**). At concentrations below 10 ppm its value declines to 25 and 30 in male and female rats, respectively, and 22 in rabbits (**Tables 1 and 2**). It was used to calculate the respective concentration in the animal (**Fig. 6**). The clearance of uptake was remarkably high, it exceeded the alveolar ventilation and approximated the pulmonary ventilation given for these species (Guyton 1947, Arms and Travis 1988) (**Tables 1 and 2**).
Only small amounts of unchanged 2-NP were exhaled as calculated from the ratio of the clearance of exhalation to the clearance of uptake: 0.13 in rabbits and male rats and 0.18 in female rats.

3.2. Metabolism of 2-NP

After the enrichment phase, the concentration-time courses of 2-NP in the atmosphere in the closed chamber (**Fig. 3a and b, Fig. 4a and b**) are predominated by the metabolic elimination. The toxicokinetic parameters of the metabolism obtained by fitting calculated curves to the experimental data are given in **Tables 1 and 2**. Calculated rates per kg body weight of the total metabolism are given in **Fig. 7.** Total metabolism in male and female rats was similar until 200 ppm 2-NP. In rabbits, corresponding values were about 2/3 of those in rats. Below 10 ppm 2-NP, the percentage of the amount metabolized to the amount taken up was between 83-89 %, the pulmonary retention was calculated to be 83-92 %.
The excellent fits to the measured data for both species and sexes were reached by describing the metabolic rate factor k_{el}^* as a function of the

concentration of 2-NP in the body (**see Fig. 1**). This function is composed of two different metabolic pathways:
- a saturable metabolism according to Michealis-Menten kinetics with low capacity and high affinity;
- a non-saturable metabolism according to first-order kinetics with the rate constant k_{elns}.

The saturable metabolism revealed striking sex differences in rats as indicated by the respective values of the maximal metabolic rate V_{max} and the apparent Michaelis-Menten constant K_{mapp} at $V_{max}/2$ (**Table 1**): in female rats V_{max} was 2.3 times and K_{mapp} (related to the concentration in the body) was 3.2 times higher than in male rats. At steady-state, the atmospheric concentration of 2-NP at $V_{max}/2$ was 71 ppm in females and 28 ppm in males. In rabbits, no sex difference was observed. If related to one kg body weight, V_{max} in rabbits was similar to the corresponding value in female rats. The rate constant k_{elns} of the non-saturable metabolism was quite similar in both sexes and also in both species.

Calculated rates per kg body weight of the two different metabolic processes in male and female rats and in rabbits are shown in **Fig. 8**. A large sex difference becomes obvious even at low concentrations if the shares of the saturable and the non-saturable pathways are shown separately. Due to the saturable pathways, the solid lines giving the share of the non-saturable pathways are curved like hockey sticks. In male rats more 2-NP is metabolized by the non-saturable than by the saturable pathway already at concentrations above 60 ppm, in female rats at concentrations above 180 ppm, and in rabbits only at concentrations above 300 ppm.

In order to inhibit cytochrome P450-dependent metabolism, experiments were performed with rats which were pretreated by i.p. injection of dithiocarb (200 mg/kg). From the beginning, the slopes of the concentration-time courses were lower compared to non-treated controls (**Fig. 9**) which was due to an inhibition of 2-NP uptake by 51 %. The most likely explanation for these findings is a dramatic reduction in breathing rate due to combination effects of 2-NP with dithiocarb. These data have therefore not been used for kinetic analysis.

In rabbits, the share of the saturable metabolism which depended on the cytochrome P450 was completely inhibited by i.p. pretreatment with pyrazol (320 mg/kg) (**Fig. 10**).

The possible occurrence of a first-pass effect due to metabolism in the liver was investigated by i.p. administration of 2-NP to female and male rats at doses of 1.7 mmol/kg (males and females) and 0.17 mmol/kg

(females). Immediately thereafter, the animals were placed into the closed chambers and the concentration-time courses of exhaled 2-NP were measured in the atmosphere of the chambers. Comparison of the measured data with curves predicted from the inhalation studies by means of the two-compartment model yielded first-pass effects of 24.5 % in females (22 % for the low and 27 % for the high dose) and of 15 % in males (**Fig. 11A and B**).

3.3. Rat Liver foci bioassay after exposure to atmospheric 2-NP

A: Suckling rats, 4-5 days old

At all exposure concentrations animals did not show any signs of sickness. Body weight gain was not impaired, and liver weight was only slightly, but non-significantly enhanced as compared to controls.

The exposure of rats to 2-NP with subsequent treatment with Clophen A50 induced ATPase-deficient preneoplastic foci in a dose-dependent manner (**Fig. 12A and B**). The number of foci/cm^2 was significantly different from controls at all concentrations in females and at concentrations higher than 40 ppm in males. The resulting concentration-reponse curves have the shape of a hockey stick, indicating that at low concentrations the effect is relatively less pronounced than at high concentrations (**Fig. 12A**). In the semilogarithmic plot linear dose-effect curves were observed with the same slope for both sexes (**Fig. 12B**). This indicates the same initiating potency of 2-NP in female and male suckling rats. In contrast, the promoter was given when the rats were 5 to 13 weeks old and became mature. Control rats with promotion only showed a foci number of 0.19/cm^2 of liver section for male and 0.73/cm^2 for females. The lesser susceptibility of adult male rats towards the promoting stimulus of Clophen A50 (Deml and Oesterle 1982) may explain that male rats exhibited a significant, about four-fold lower foci incidence at all exposure concentrations of 2-NP.

B: Adult rats

At all exposure concentrations, body weight, liver weight, and liver to body weight ratio were normal in all animals (data not shown).

Like in suckling rats, exposure to 2-NP and subsequent treatment with Clophen A50 induced ATPase-deficient preneoplastic foci in a dose-dependent manner (**Fig. 13A and B**), indicating that 2-NP is an initiating agent also in adult male and female rats in the bioassay used. Furthermore, the concentration-response curves had a similar shape as in suckling rats. No dose-response effect was observed with respect to foci areas (data not shown). Control rats with promotion only showed a foci number of 0.5/cm^2

of liver section for males and 1.2/cm^2 for females. The number of foci/cm^2 was significantly different from controls at all concentrations in males and at concentrations higher than 25 ppm in females. Unlike in suckling rats, the slopes of the concentration-response curves in the semilogarithmic plot are different for both sexes. The slope obtained with the data of males is equal to that obtained with the data of suckling animals, whereas the slope obtained with the data of females is only half that value.

3.4. Liver enzymes in serum of rats after single i.p. doses of 2-NP

2-NP, 1.7 mmol/kg b.w., was administered i.p. to female and male rats. After eight hours, the liver enzymes GOT, GPT, and OCT, used as markers for acute liver damage, reached peak concentrations in blood serum (**Fig. 14**). The values in females were 1.9-fold (GOT), 2.7-fold (GPT), and 7.9-fold (OCT) higher than in controls. In males the corresponding values were 22-fold (GOT), 20-fold (GPT), and 460-fold (OCT) higher than in controls. The dose-response relationship of i.p. administered 2-NP and of liver enzymes was investigated in male rats at concentrations between 0.13 and 3.4 mmol/kg. Typical dose-response curves were found using the enzyme activities as parameters (**Fig. 15**). In every case the maximum effect was reached after dosing 1.7 mmol/kg 2-NP.

3.5. Software for a sophisticated toxicokinetic analysis

A program "SOLVEKIN" was developed which is able to solve special toxicokinetic problems as simulation and parameter estimation for data representing a single curve or a set of curves. These curves can be described by functions or by first order differential equations.

SOLVEKIN computes the kinetic parameters using the method of the least error squares and the simplex algorithm of Nelder and Mead (1965) for function minimization. To obtain the confidence intervals of the estimated parameters, the curvature of the sum-squared error function is examined near the minimum. The first order differential equations are solved by different methods (Runge-Kutta, Adams-Moulton, Bulirsh-Stoer) with automatic stepsize control. Sets of curves are treated as a surface and are fitted simultaneously. The program was written in C, because it allows a dynamic programming style and makes porting easy. It can be compiled without further changes in the source under VMS, UNIX, MSDOS, MAC OS, and MIPS OS.

4. DISCUSSION

4.1. Determination of the inhalation kinetics

The closed chamber technique (CCT) is a suitable tool to study the enrichment and the metabolism of gases and vapors *in vitro* and *in vivo* (Filser 1992). The decline of the concentration-time course in the atmosphere of the chamber after a single administration of the substance at the beginning of the exposure can be measured directly by gas chromatography. The toxicokinetic evaluation of such concentration-time courses by a two-compartment model provides informations upon the enrichment of the substance in the body as whole and upon rates of uptake, of exhalation and of metabolic elimination. With different starting concentrations a set of concentration-time courses is obtained which allow an exact determination of the toxicokinetic parameters of the processes mentioned above. These parameters obtained for steady-state conditions in a closed system can easily be converted for an open system with an infinitely large atmospheric volume which results in a constant exposure concentration. Mathematical details of such toxicokinetic evaluations are reviewed in Filser (1992). The parameters describing inhalative uptake, exhalation and metabolic elimination are given as clearances [ml/h] being a measure of the rate of the respective process. Their values multiplied with the actual atmospheric concentration of 2-NP give its amount taken up, exhaled or metabolized per hour.

2-NP showed a similar kinetic behavior in rats and in rabbits. The thermodynamic partition coefficient body/air (K_{eq}) depending solely on the solubility of the substance was nearly equal in both species. From the good solubility of 2-NP in both, olive oil and water, it can be concluded that 2-NP distributes nearly homogeneously within the body. The clearance of uptake approximated the pulmonary ventilation. This indicates a complete extraction by the respiratory system and probably by the skin. Furthermore, rearrangement of 2-NP to its nitronate anion and propane-2-nitronic acid, both of which have been shown in aqueous systems (Porter and Bright 1983), may favor removal from the air in the airways by accumulation in the respiratory epithelium (Johanson and Filser 1992).
Maximal values of the clearance of metabolism, i.e. 82-87 % of the clearance of uptake, were reached below 10 ppm 2-NP. At these concentrations the clearance of metabolism is obviously limited by ventilation. Consequently, the bioaccumulation body/air below this concentration had values only between 22 and 30 compared to the thermodynamic partition coefficient body/air having values between 170

and 180. Up to atmospheric concentrations of 200 ppm, relatively small differences of the concentrations of unmetabolized 2-NP in the animals were found between sexes and species. Furthermore, the total amount of 2-NP metabolized per kg body weight was similar in male and female rats and only slightly lower in rabbits. The non-saturable pathway was also comparable in male and female rats and in rabbits. Its half-life (2.1-3.85 h) was similar to that found *in vitro* for the conversion from 2-NP into its nitronate anion at pH 7 (2.5 h, Nielsen 1969, Porter and Bright 1983).

Marked differences between male and female rats and between rats and rabbits, however, occurred due to the characteristics of the saturable metabolic pathway. This pathway has already been described by Nolan et al. (1982) in male rats exposed to 20 and 154 ppm of 2-NP. Related to body weight, the maximal capacity Vmax of this pathway is similar in female rats and in rabbits, but lower in male rats. As indicated by the complete inhibition of this pathway by pyrazol in rabbits, the responsible enzyme can assumed to be the alcohol-oxidizing cytochrome P450 3a (Koop et al. 1985). Over the whole concentration range the share of the saturable pathway in the total metabolism of female rats and rabbits was much higher than in male rats, whereas the share of the non-saturable pathway in the total metabolism was highest in male rats and lowest in rabbits.

4.2. Hepatocarcinogenicity of 2-NP

Carcinogenicity of inhaled 2-NP has been tested in long-term bioassays with rats and rabbits. Results of these bioassays were available only for exposure concentrations of about 25 and 200 ppm (Lewis et al. 1979, Griffin et al. 1980, 1981). To evaluate the carcinogenic potency of 2-NP in the lower concentration range we performed a short-term carcinogenicity test at 5 exposure concentrations between 25 and 150 ppm. The rat liver foci bioassay was chosen because of its high sensitivity and the possibility to distinguish between initiating and promoting substances (Oesterle and Deml 1990). The incidence of the enzyme-altered foci in the liver represents a quantitative measure for hepatocarcinogenicity (Kunz et al. 1978).
This assay has been successfully used in our institute with liver carcinogens like diethylnitrosamine (Deml et al. 1981), but also with non-liver carcinogens like ethylene oxide (Denk et al. 1988). The applied initiation-promotion protocol using polychlorinated biphenyls as a promoter has advantages over protocols using partial hepatectomy or initiation-selection protocols using cytotoxic agents like acetaminofluorene (Oesterle

et al. 1989). Of these test protocols it is the most sensitive and causes the lowest impairment of animals welfare.
The results obtained with suckling and adult rats confirmed the outcome of the carcinogenicity studies demonstrating the higher sensitivity of male compared to female rats (Griffin et al. 1980). Beyond it, the development of the sex-specific carcinogenic potency of 2-NP during the maturing of the animals can also clearly be observed from the obtained data. The linearity of the concentration-effect curves in the semilogarithmic plot indicates that 2-NP has no threshold as could be concluded considering solely the long-term studies.

The mechanism leading to hepatocarcinogenicity of 2-NP has not yet been fully elucidated. Griffin et al. (1980) and Cunningham and Matthews (1991) proposed an epigenetic mechanism based on hepatocellular proliferation due to the hepatotoxic effects of 2-NP. These effects of 2-NP are very similar to those observed with the hepatocarcinogen carbon tetrachloride (see below).
Other investigations have been performed to find certain metabolites which could be related to the carcinogenic potential of 2-NP. Metabolic pathways leading to proposed carcinogenic metabolites of 2-NP are shown in **Fig. 16.** Nitrite was formed in liver microsomes of rats and mice together with acetone by an oxidative denitrification of 2-NP (Ullrich et al. 1978, Marker and Kulkarni 1985, 1986, Dayal et al. 1991) and of the nitronate of 2-NP (Dayal et al. 1991). Nitrite could react with primary amino groups to form carcinogenic nitrosamines. However, the generation of nitrite in uninduced microsomes was very low. Furthermore, the liver specificity of 2-NP excludes nitrite to be the ultimate carcinogen because it was found also in other organs which are not affected by 2-NP (Dequindt et al. 1972).
Several groups (Fiala et al. 1987b, 1989, Guo et al. 1990, Hussain et al. 1990, Dayal et al. 1989, Haas-Jobelius et al. 1990, Roscher et al. 1991) have recently tried to find the origin of the high genotoxic potential of 2-NP. Haas-Jobelius et al. (1991) investigated the nitroreduction of 2-NP to acetone oxime, the tautomeric form of 2-nitrosopropane, to isopropyl hydroxylamine and to 2-aminopropane in primary rat hepatocytes and V79 cells. They found no relationship between this metabolic pathway and genotoxic effects of 2-NP. Roscher et al. (1991) observed genotoxic effects of 2-NP in various hepatoma cell lines after the induction of cytochrome P450, but also in V79 cells where no cytochrome P450 was expressed. They conclude that different pathways exist leading to metabolites with a genotoxic potential.

Fiala et al. (1987b) and Dayal et al. (1989) compared the mutagenicity of 2-NP and its nitronate in *S. typhimurium*. Both groups found the nitronate to be the more powerful mutagen. Fiala et al. (1987b, 1989) and Hussain et al. (1990) reported oxidative damage of bases in DNA and RNA of bacteria and of the liver of rats exposed to 2-NP. Guo et al. (1990) investigated a possible sex difference of such oxidative DNA and RNA damage in the liver of rats. They found 2-4fold higher values in males than in females.
The mechanism by which the nitronate leads to such damages might be the further oxidation to a free nitronate radical. This process may proceed enzymicatically (Porter and Bright 1983, Kido et al. 1984) or non-enzymicatically (Kuo and Fridovich 1986). The radical formed might be able to oxidize nucleotides directly or indirectly via autoxidation and subsequent production of oxygen radicals.

4.3. Hepatotoxicity of 2-NP

Our results concerning the hepatotoxicity of 2-NP in rats with a maximum effect at 8 h after i.p. administration of 1.7 mmol/kg 2-NP demonstrates the high sensitivity of males compared to females. From the dose-response curve in males rats it can be observed that the effect increased dramatically above 0.9 mmol/kg. Cunningham and Matthews (1991) treated male Fisher F-344 rats i.p. with 0.23-0.9 mmol/kg and found only slight signs of hepatotoxic effects at the highest concentration. Zitting et al. (1981) described hepatotoxic effects after i.p. administration of 0.56 mmol/kg 2-NP in male rats which were similar to the effects induced by carbon tetrachloride and more pronounced after 24 h than after 4 h. In mice, hepatic enzymes in serum were elevated only 48 h after i.p. administration of 6.7 mmol/kg to females and 9 mmol/kg to males (Dayal et al. 1989). Zitting et al. (1981) assumed that the toxic effects were due to oxidative damage of membrane structures as has been found for carbon tetrachloride (Recknagel and Glende jr 1974). The authors proposed a nitroreductase-dependent formation of a nitro-anion free radical which is able to reduce oxygen to radical species. At the moment, the nitronate is favored to be the most relevant metabolite of 2-NP leading to hepatotoxicity. Linhart et al. (1991) investigated if the difference in the hepatotoxicity of 2-NP and 1-NP could be due to the respective nitronates. After equilibration at physiological pH half of initial propane 2-nitronate existed unchanged whereas only a small part of the 1-isomer remained in the nitronate form. The authors concluded that this difference is probably an important chemical determinant of the respective toxic potential. Dayal et al. (1991) investigated the hepatotoxicity of 2-NP and of the nitronate of 2-NP *in*

vitro using freshly isolated hepatocytes from mouse and rat: No difference in the cytotoxicity of both compounds was found. The nitronate, however, was metabolized 5-10 times more rapidly than 2-NP itself by an NADPH-dependent metabolic pathway in liver microsomes isolated from mice pretreated with phenobarbital. The authors concluded that this pathway could not be responsible for the observed similar cytotoxicity and that a different metabolic route was involved. On the other hand they admitted hepatocyte suspensions to be a wrong model, because there was no difference in hepatocytotoxicity of 2-NP, nitromethane and nitroethane. In male mice only 2-NP caused necrosis in the liver. The lack of difference *in vitro* might be caused by the short time span during which irreversible lesions could not manifest.

4.4. Correlation of the inhalation toxicokinetics with the hepatocarcinogenicity and hepatotoxicity of 2-NP

From our toxicokinetic data obtained in male and female rats and in rabbits we conclude, that the differences in carcinogenicity and hepatotoxicity found in female and male rats and in rabbits cannot be attributed to unmetabolized 2-NP, because its enrichment from the atmosphere into the body at steady-state conditions is nearly equal in female and male rats and in rabbits (**Figs. 5 and 6**). The liver specificity of the toxic and carcinogenic effects induced by 2-NP is also an indication against a direct action of 2-NP itself. Due to the good solubility in water and fat the substance distributes nearly homogeneously in the whole organism and should induce effects in other organs too. The total metabolism of 2-NP was also concluded not to be responsible for these effects because the difference between female and male rats and rabbits was not large enough to correlate with the divergent effects of 2-NP in these animals.

Only the different values of the the non-saturable pathway of 2-NP can explain the observed sex and species specific differences. This becomes obvious by comparing the concentration-effect curves of the rat liver foci bioassay (adult animals) with the concentration-metabolic rate curves of the two metabolic pathways of 2-NP. The number of foci/cm^2 were plotted against the exposure concentrations of 2-NP **(Fig. 17A and B)**. The lines in these figures represent the shares of saturable and of non-saturable metabolic elimination as already shown in **Fig. 8**. Only the hockey stick-like shapes of the curves, which describe the concentration dependence of the non-saturable metabolic pathway, match the concentration dependence of the foci incidences in both sexes.

These correlations and the relatively higher share of the non-saturable pathway in male rats together with their higher sensitivity as compared to females support the hypothesis that carcinogenic and toxic effects of 2-NP are probably based on the non-saturable pathway (Denk et al., 1989). In contrast, the saturable pathway is more likely to lead to less toxic or carcinogenic metabolites, since at identical concentrations of 2-NP this pathway was slower in males than in females.

In rabbits, the non-saturable pathway of 2-NP was even slower than in female rats, explaining the lack of tumors at an exposure concentration of 200 ppm.

In both sexes and species, no liver tumors were observed at 25 ppm. This can be rationalized even for male rats, since at concentrations below 60 ppm the saturable pathway overwhelms the non-saturable one.

From the hockey stick-like shape of the foci incidence and the non-saturable pathway we conclude that no threshold concentration of the hepatocarcinogenic action of 2-NP exists, although the risk of hepatocarcinogenicity should be relatively lower at low exposure concentrations of 2-NP.

The half-lives of the non-saturable pathway were similar to the half-life of the conversion of 2-NP to this nitronate anion at pH 7 (see above). Therefore, it becomes plausible that the non-saturable pathway represents this conversion. As discussed above, recent investigations *in vitro* and *in vivo* support the hypothesis that the hepatotoxic and hepatocarcinogenic effects proceed from the formation of this metabolite.

4.5. Toxicokinetics and species specificity of 2-NP

The non-saturable pathway showed similar rate constants in rats and rabbits. The metabolic rates of this pathway were proportional to body weight, which becomes understandable assuming that the non-saturable pathway presents a non-enzymatic conversion of 2-NP to its nitronate anion. However, the saturable pathway depended on sex and species specific enzyme capacities. Considering only the difference in body weight, the maximum rate (V_{max}) should be about 10 times higher in rabbits as compared to rats. This extrapolation holds true if female rats and rabbits are compared (**Table 3**).

Although kinetics of the metabolism of 2-NP related to the concentration within the body are quite similar in female rats and rabbits the concentration-metabolic rate curves of the non-saturable pathway differ markedly if related to the exposure concentration in the atmosphere. Whereas the maximum metabolic rate was to the body weight, rates of

inhalation and exhalation seemed to be limited by the air way surface. Physiological processes depending on surfaces can be scaled from one species to another using the factor (body weight)$^{2/3}$ (Filser 1992). This 'surface factor' was calculated to be 4.64 between a rat and a rabbit; the respective body weight factor was 10. Therefore, at a given concentration of 2-NP the ratio 'amount taken up/amount metabolized' is smaller in rabbits. Consequently, the bioaccumulation at steady state, K_{st}, becomes smaller, too. A result of this difference in bioaccumulation is that at equal atmospheric concentrations of 2-NP, a relatively lower amount was metabolized by the non-saturable pathway in rabbits as compared to rats **(see Fig. 8)**. This signifies that rabbits should be less sensitive to inhaled 2-NP, if, as assumed above, carcinogenic effects of 2-NP are based on the non-saturable pathway. The negative effect of 2-NP in the carcinogenicity test corroborates this assumption.

Based on this conclusions, a cautious estimation of the risk for man can be done. The ratio 'body surface/body weight' becomes smaller with increasing body weight. For man, a bioaccumulation factor of 5 below concentrations of 10 ppm 2-NP is calculated as described above from the female rat. If kinetics of the saturable pathway are similar in both species, man should metabolize much more 2-NP by this pathway. Consequently, the share of 2-NP metabolized by the non-saturable pathway and therefore the tumor risk by 2-NP should be much smaller in man.

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Table 1. Toxicokinetic parameters of 2-NP in male and female rats

Parameter		Value		Dimension
Name	Expression	Males (250 g)	Females (250 g)	
Thermodynamic equilibrium constant (body/air)	K_{eq}	180	180	nl gas/ml tissue / ppm in atmosph.
Bioaccumulation factor [a] (body/air)	K_{st}^*	23	30	nl gas/ml tissue / ppm in atmosph.
Clearance of uptake [a] (related to atmosph. conc.)	$k_{12}\ V_1$	10,800	10,800	ml/h
Clearance of exhalation [a] (related to atmosph. conc.)	$k_{21}\ V_2\ K_{st}^*$	1,440	1,920	ml/h
Clearance of metabolism [a] (related to atmosph. conc.)	$k_{el}^*\ V_2\ K_{st}^*$	9,600	9,000	ml/h
share of the non-satur. metab. [a]	$k_{elns}\ V_2\ K_{st}^*$	1,900	1,500	ml/h
share of the satur. metab. [a]	$\frac{V_{max}\ K_{st}^*}{K_{mapp}/V_{mol}}$	7,700	7,500	ml/h
Half-life of the total metab. [a]	$\ln 2/(k_{el}^*+k_{21})$	0.36	0.48	h
Half-life of the non-satur. metab.	$\ln 2/k_{elns}$	2,1	3,5	h
Amount metabolized · 100 / Amount taken up [a]	$\frac{k_{el}^*\ 100}{k_{el}^* + k_{21}}$	87	82	%
Amount exhaled · 100 / Amount taken up [a]	$\frac{k_{21}\ 100}{k_{el}^*+k_{21}}$	13	18	%
Pulmonary retention [a], [b]	$\frac{k_{el}^*\ V_2\ K_{st}^*\ 100}{\text{pulm. ventilation}}$	89	83	%
Maximum rate of metabolism	V_{max}	12.6	28.8	µmol/h
Apparent Michaelis-Menten constant	K_{mapp}	900	2,900	nl gas/ml tissue

[a] Values are valid for atmospheric concentrations up to 10 ppm.

[b] Pulmonary ventilation was set to 10,800 ml/h (Arms and Travis 1988).

Table 2. Toxicokinetic parameters of 2-NP in male and female rabbits

Parameter		Value	Dimension
Name	Expression	body weight: 2,500 g	
Thermodynamic equilibrium constant (body/air)	K_{eq}	170	$\frac{\text{nl gas/ml tissue}}{\text{ppm in atmosph.}}$
Bioaccumulation factor [a] (body/air)	K_{st}^*	22	$\frac{\text{nl gas/ml tissue}}{\text{ppm in atmosph.}}$
Clearance of uptake [a] (related to atmosph. conc.)	$k_{12}\ V_1$	62,000	ml/h
Clearance of exhalation [a] (related to atmosph. conc.)	$k_{21}\ V_2\ K_{st}^*$	8,300	ml/h
Clearance of metabolism [a] (related to atmosph. conc.)	$k_{el}^*\ V_2\ K_{st}^*$	53,400	ml/h
share of the non-satur. metab. [a]	$k_{elns}\ V_2\ K_{st}^*$	9,900	ml/h
share of the satur. metab. [a]	$\frac{V_{max}\ K_{st}^*}{K_{mapp}/V_{mol}}$	43,500	ml/h
Half-life of the total metab. [a]	$\ln 2/(k_{el}^*+k_{21})$	0.62	h
Half-life of the non-satur. metab.	$\ln 2/k_{elns}$	3.85	h
$\frac{\text{Amount metabolized} \cdot 100}{\text{Amount taken up}}$ [a]	$\frac{k_{el}^*\ 100}{k_{el}^* + k_{21}}$	86.6	%
$\frac{\text{Amount exhaled} \cdot 100}{\text{Amount taken up}}$ [a]	$\frac{k_{21}\ 100}{k_{el}^*+k_{21}}$	13.4	%
Pulmonary retention [a], [b]	$\frac{k_{el}^*\ V_2\ K_{st}^*\ 100}{\text{pulm. ventilation}}$	92	%
Maximum rate of metabolism	V_{max}	315	µmol/h
Apparent Michaelis-Menten constant	K_{mapp}	4,000	nl gas/ml tissue

[a] Values are valid for atmospheric concentrations up to 10 ppm.

[b] Pulmonary ventilation was set to 58,000 ml/h (Guyton 1947).

Table 3. Comparison of toxicokinetic parameters of 2-NP in a female rat (250 g) and in a rabbit (2,500 g). For description of the parameters see Fig. 1.

Parameter		female rat (liver weight 10 g)	rabbit (liver weight 100 g)		
			extrapolated	observed	extrap./obs. [%]
K_{st}^{*} [a]	nl gas/ml tissue / ppm in atmosph.	30	15	22	69
$k_{12}\ V_1$	ml/h	10,800	50,000	62,000	81
k_{21}	h^{-1}	0.26	0.12	0.15	80
k_{el}^{*} [a] [b]	h^{-1}	1.2	1.2	0.97	124
k_{elns}	h^{-1}	0.2	0.2	0.18	111
V_{max}	µmol/h	28.8	290	315	92
K_{mapp}	nl gas/ml tissue	2,900	2,900	4,000	73

[a] Values are valid for atmospheric concentrations up to 10 ppm.

[b] When it is assumed that the non-saturable and the saturable metabolism are proportional to the liver weight, then k_{el}^{*} should be equal in both species.

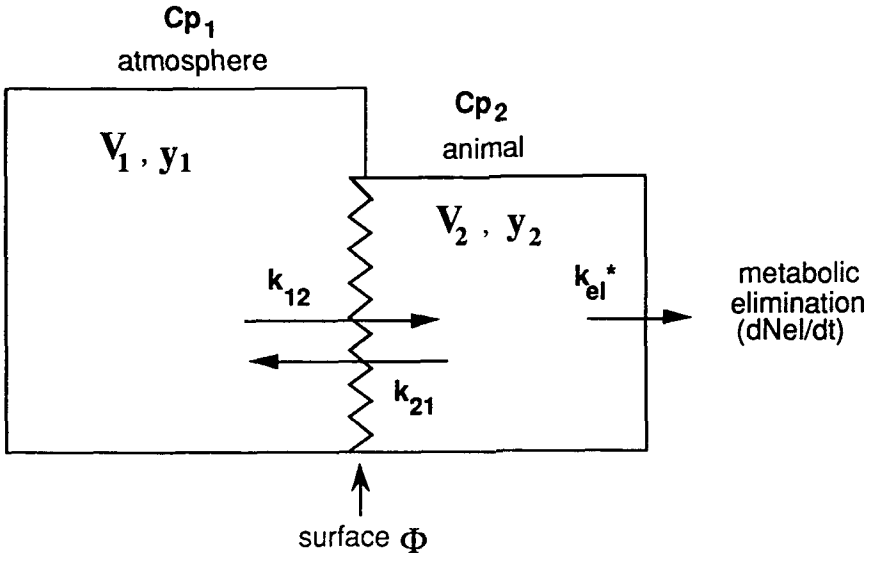


Fig. 1. Toxicokinetic two-compartment model used to analyze the inhalation kinetics of 2-NP

The following differential equations were used in the two-compartment model to describe the concentration of 2-NP (y_1) in the

atmosphere: $$V_1 \frac{dy_1}{dt} = - k_{12}V_1 y_1 + k_{21}V_2 y_2$$

animal: $$V_2 \frac{dy_2}{dt} = k_{12}V_1 y_1 - (k_{el}^* + k_{21})V_2 y_2$$

Cp_1: compartment 1; Cp_2: compartment 2;
y_1: atmospheric concentration of 2-NP
y_2: concentration of 2-NP in the animal
V_1: volume of gas phase;
V_2: volume of animal
k_{12}: microconstant of uptake from the atmosphere
k_{21}: microconstant of exhalation
k_{el}^*: rate factor of the metabolism (concentration dependent)

Equation used for k_{el}^*: $$k_{el}^* = \frac{V_{max}}{V_2(Km_{app}+y_2)} + k_{elns}$$

V_{max}: maximal rate of metabolism;
Km_{app}: apparent Michaelis-Menten constant for a saturable pathway;
k_{elns}: microconstant describing first-order kinetics (non-saturable pathway)

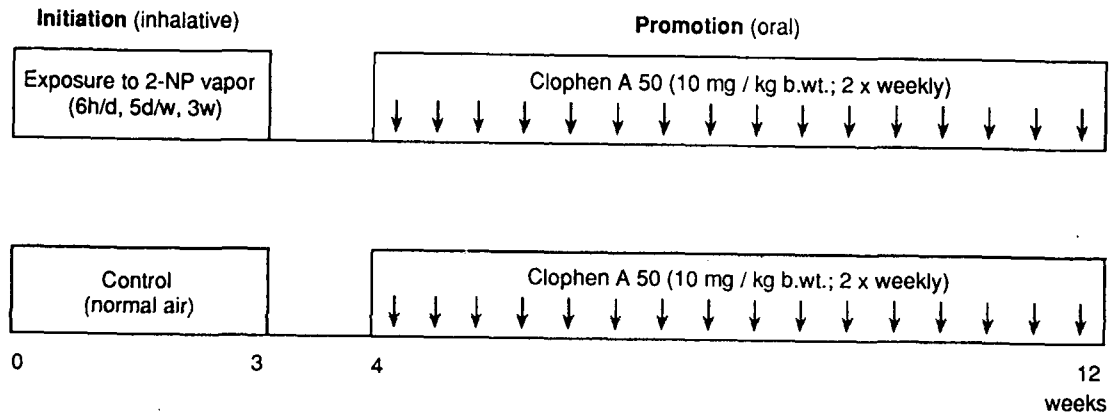


Fig. 2: Scheme of the initiation-promotion protocol used for the rat liver foci bioassay with suckling and adult animals

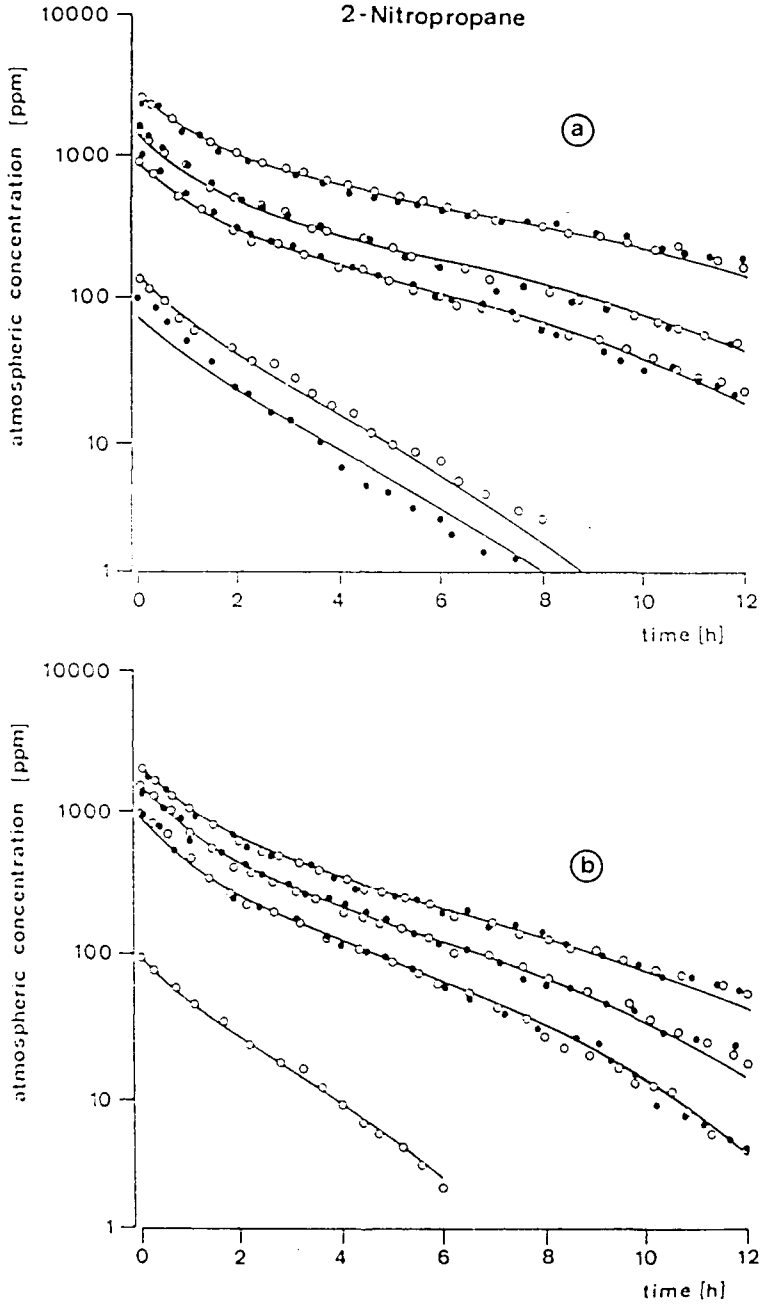


Fig. 3. Concentration-time curves of inhaled 2-NP at different initial concentrations in the gas phase of a closed exposure chamber occupied by two SD rats.
(a) females, (b) males
Dots: measured values; lines: calculated curves using the kinetic data shown in table 1.

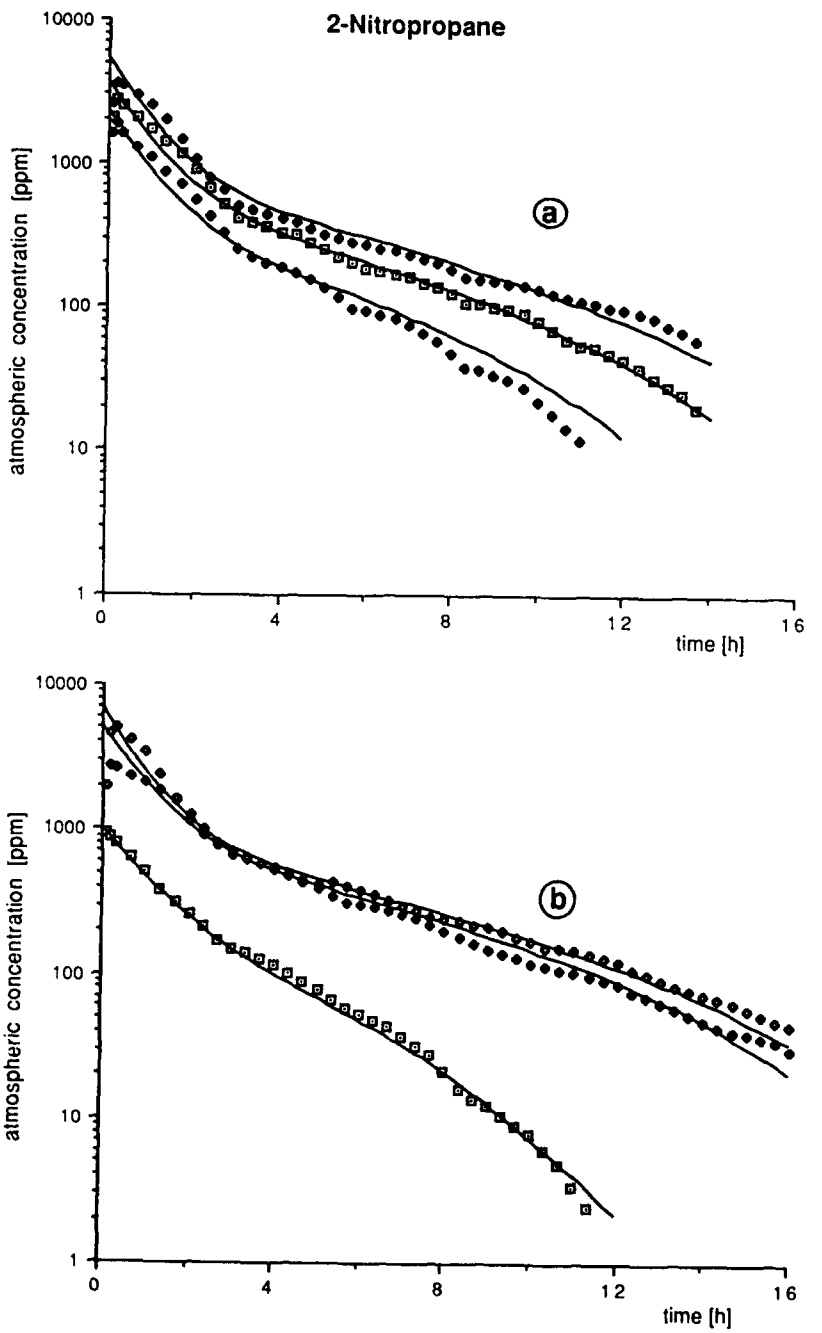


Fig. 4. Concentration-time courses of inhaled 2-NP at different initial concentrations in the gas phase of a closed exposure chamber occupied by one rabbit
(a) male, (b) female
Symbols: measured values; lines: calculated curves using the kinetic data shown in Table 2.

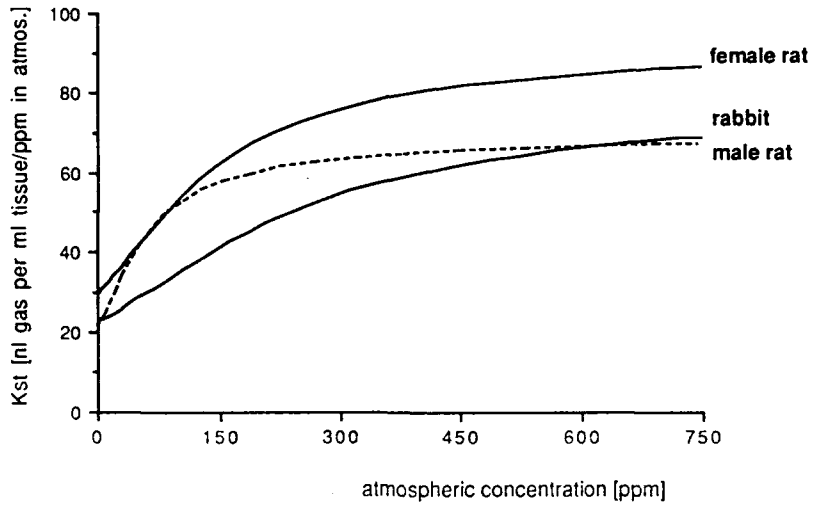


Fig. 5. Enrichment of 2-NP in the organism of rats and rabbits at steady-state conditions in dependence on the concentration of 2-NP in the atmosphere
Solid lines: female rats and rabbits; dashed lines: male rats
Kst: bioaccumulation factor body/air at steady state, calculated for a body weight of 1 kg.

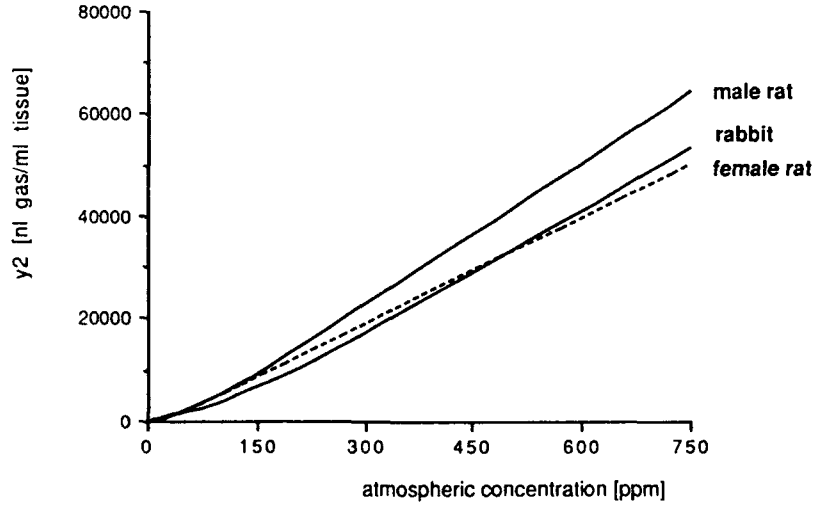


Fig. 6. Concentration of 2-NP in the animal at steady-state conditions in dependence on the concentration of 2-NP in the atmosphere
Solid lines: male rat and rabbit; dashed line: female rat

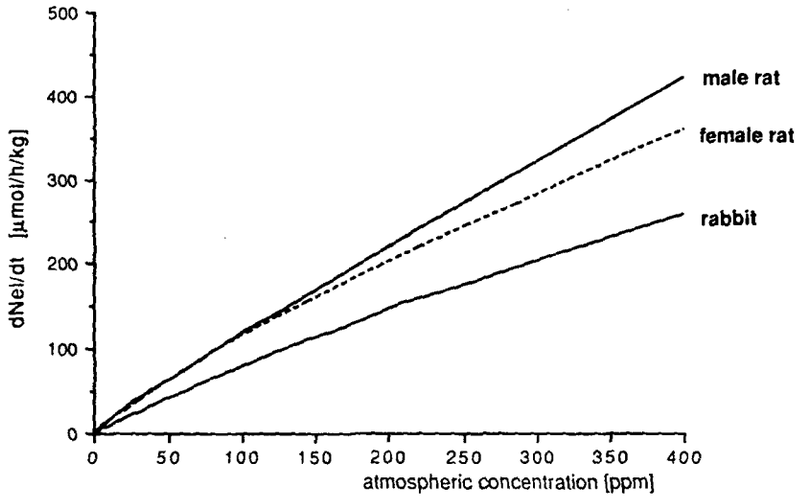


Fig. 7. Rate of total metabolism (dNel/dt) of 2-NP per kg at steady-state conditions in male and female rats and rabbits in dependence on the atmospheric concentration (calculated for an open exposure system)
Solid lines: male rat and rabbit; dashed line: female rat

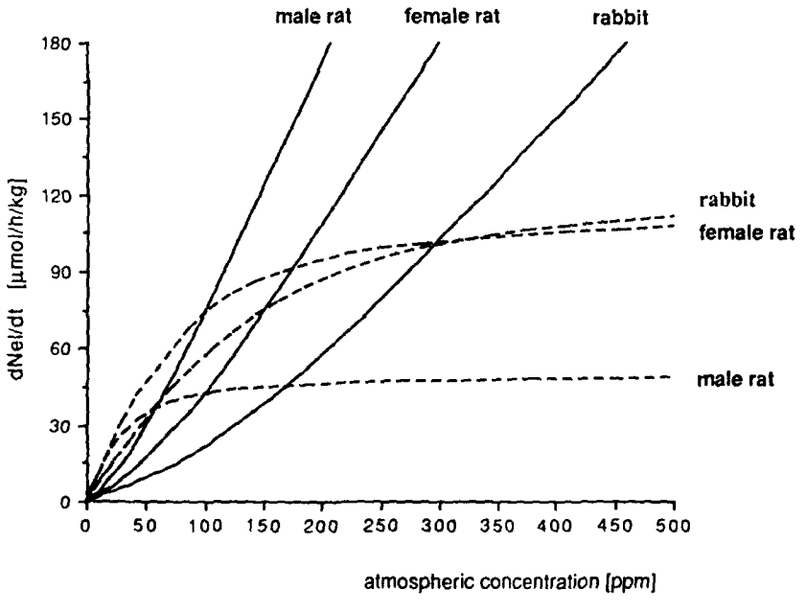
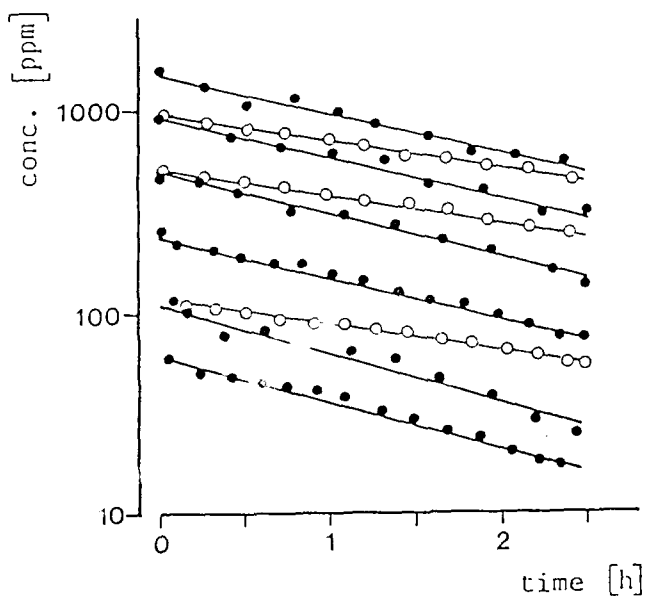


Fig. 8. Rates of the two metabolic pathways (dNel/dt) of 2-NP per kg at steady-state conditions in male and female rats and rabbits in dependence on the atmospheric concentration (calculated for an open exposure system)
Solid lines: non-saturable metabolic pathway
Dashed lines: saturable metabolic pathway

Fig. 9. Concentration-time curves of 2-nitropropane in the atmosphere of the exposure system, occupied by two rats



dots: measured; lines: calculated
●——●: untreated; o——o: pretreated with dithiocarb

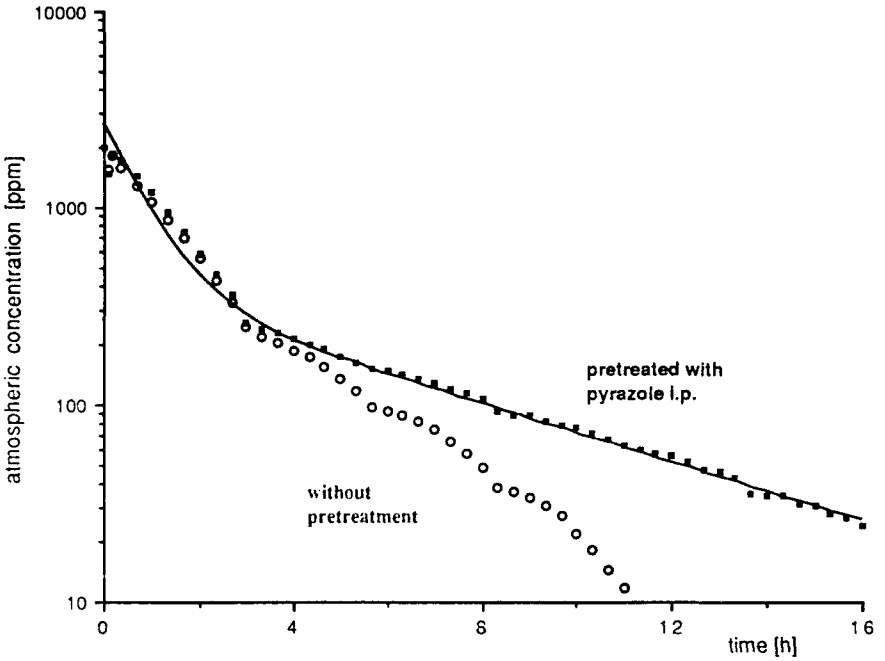


Fig. 10. Concentration-time course of 2-NP in the atmosphere of a closed exposure chamber occupied with one rabbit, either untreated or pretreated with pyrazole (320 mg/kg); initial 2- NP concentrations were identical in both experiments
Symbols: measured data
Line: calculated curve using the kinetic data shown in Table 2, assuming only the metabolic elimination according to first-order kinetics

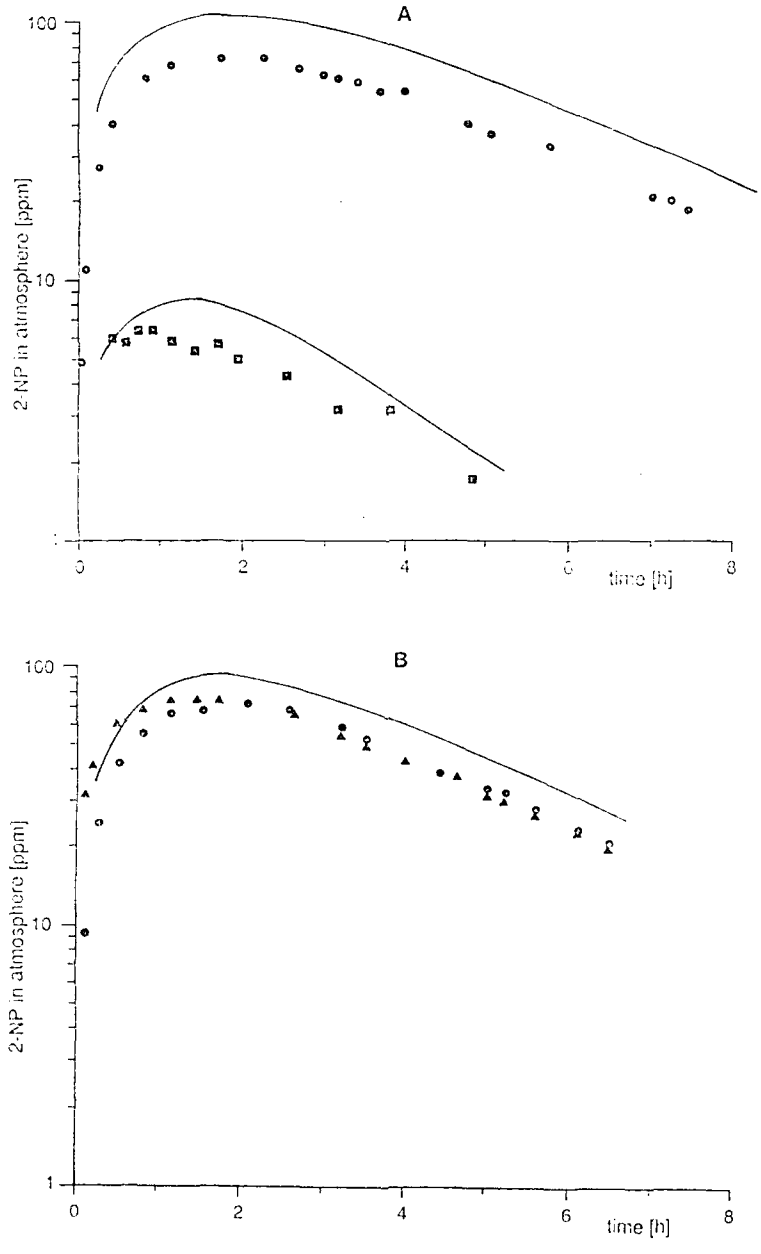


Fig. 11. Exhalation of 2-NP into the atmosphere of a closed chamber (20 l) after i.p. administration to one rat.
A: Female rat: 0.17 or 1.7 mmol/kg, one experiment each
B: Male rat:1.7 mmol/kg, two experiments
Symbols: measured values; lines: curves calculated with the toxicokinetic parameters obtained from inhalation studies assuming no first-pass effect

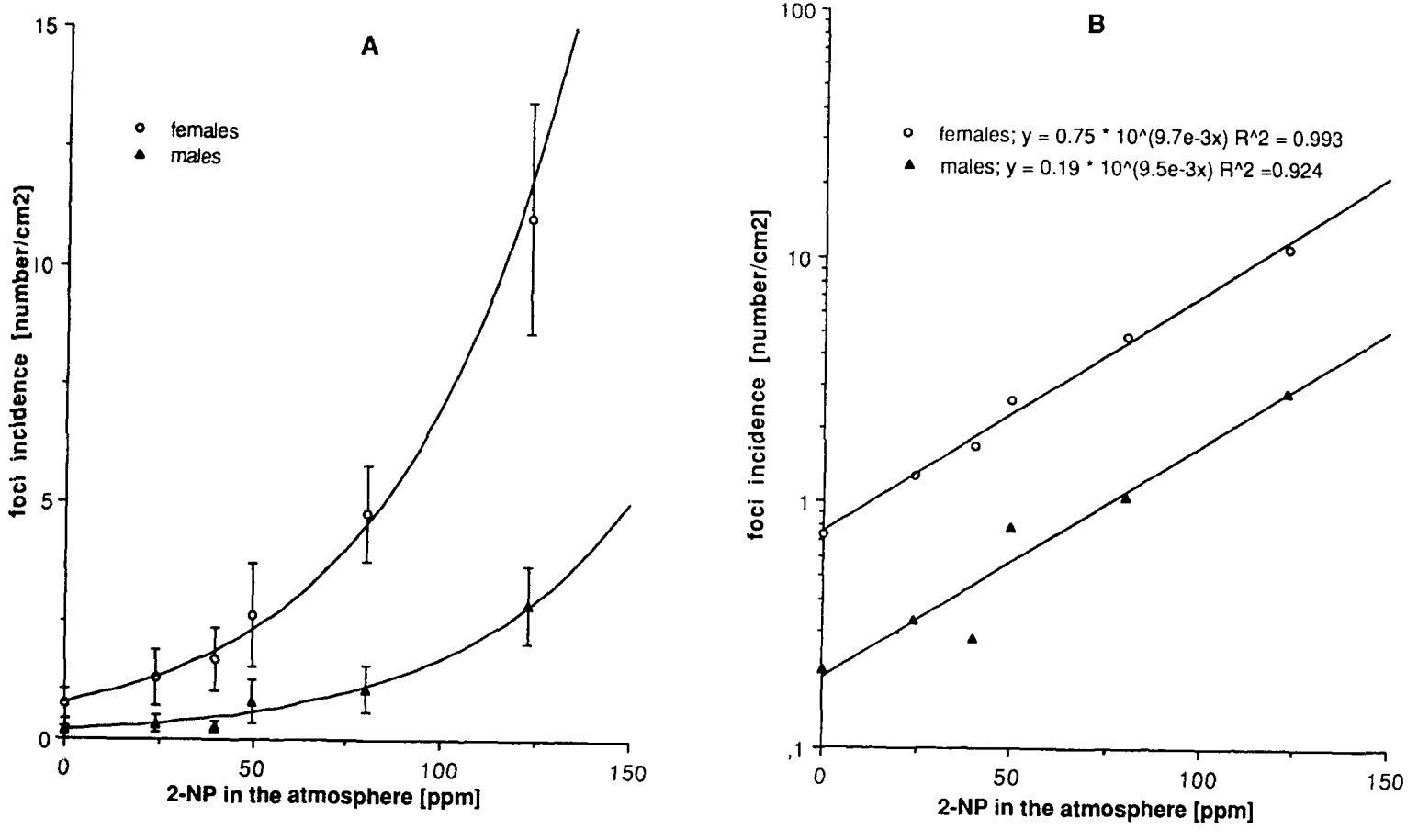


Fig. 12. Foci incidence in the liver of rats exposed as suckling animals to constant concentrations of 2-NP (6 h/d, 5d/w, 3 w) followed by treatment with Clophen A50 for 8 weeks
A: linear plot B: half-logarithmic plot

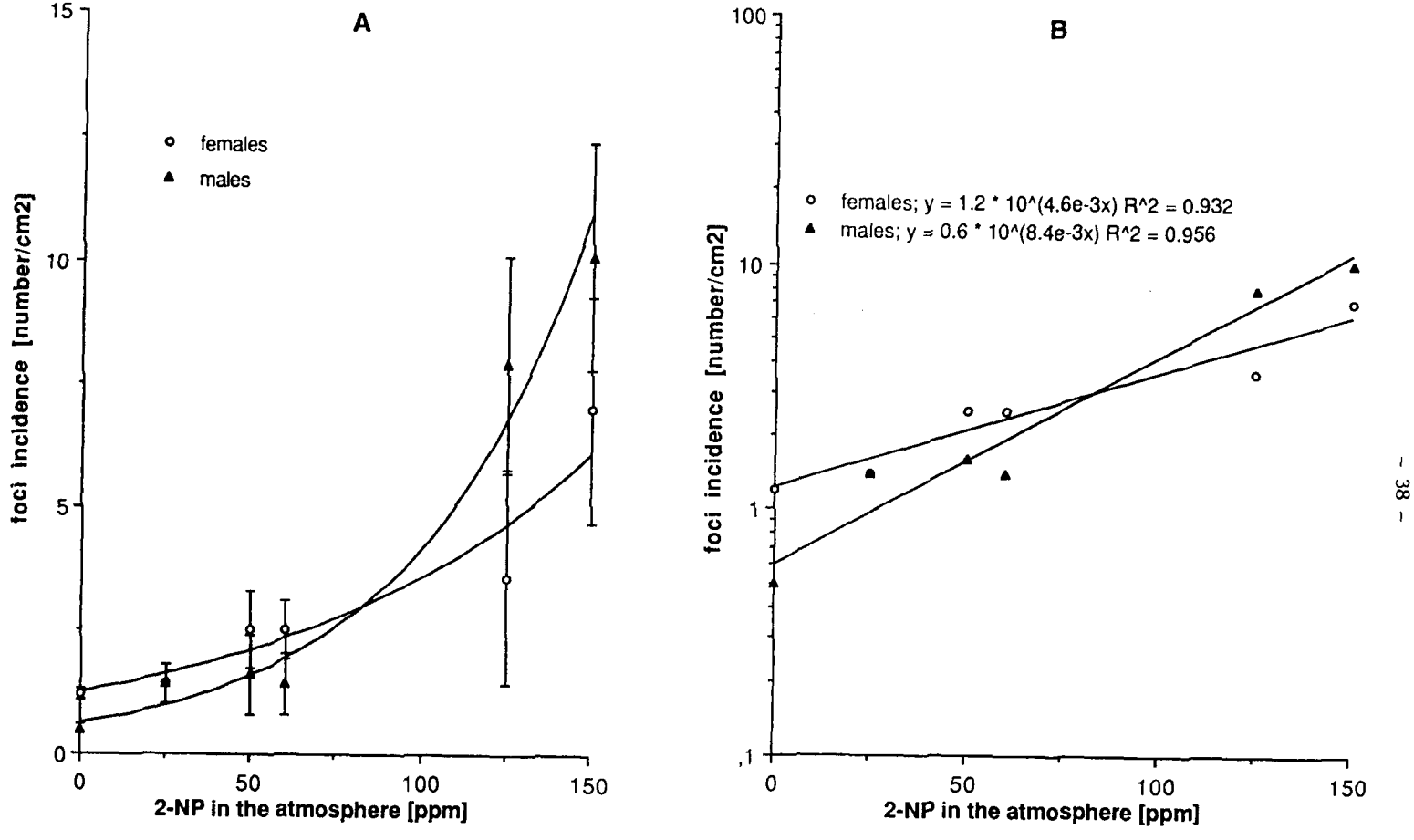


Fig. 13. Foci incidence in the liver of rats exposed as mature animals to constant concentrations of 2-NP (6 h/d, 5 d/w, 3 w) followed by treatment with Clophen A50 for 8 weeks
A: linear plot B: half-logarithmic plot

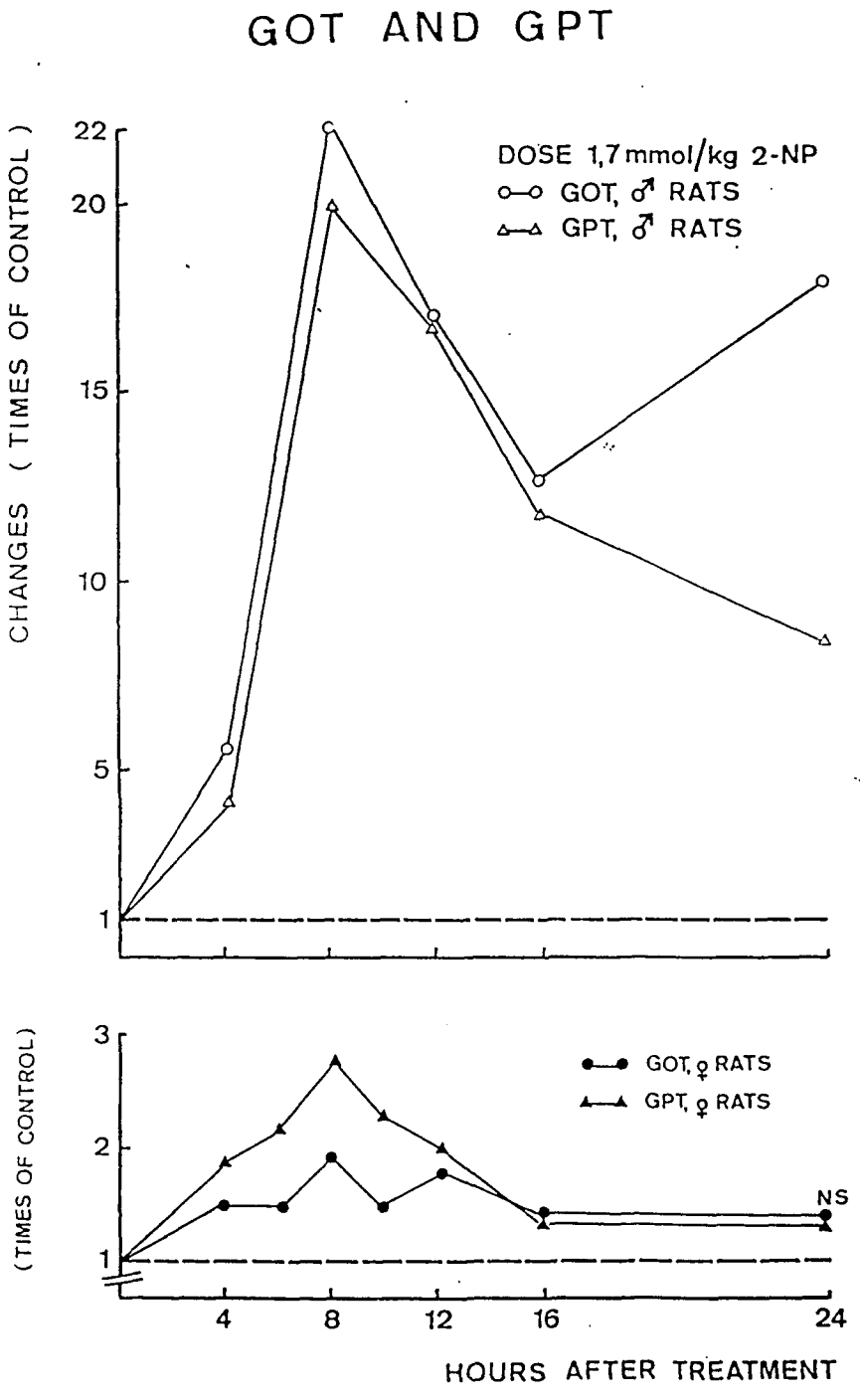


Fig. 14. Time-course of the activities of GOT and GPT in serum of female and male rats after single IP-doses of 2-NP (1.7 mmol/kg b.wt.). Data are given as multiple changes of controls. NS: not significant (n=6)

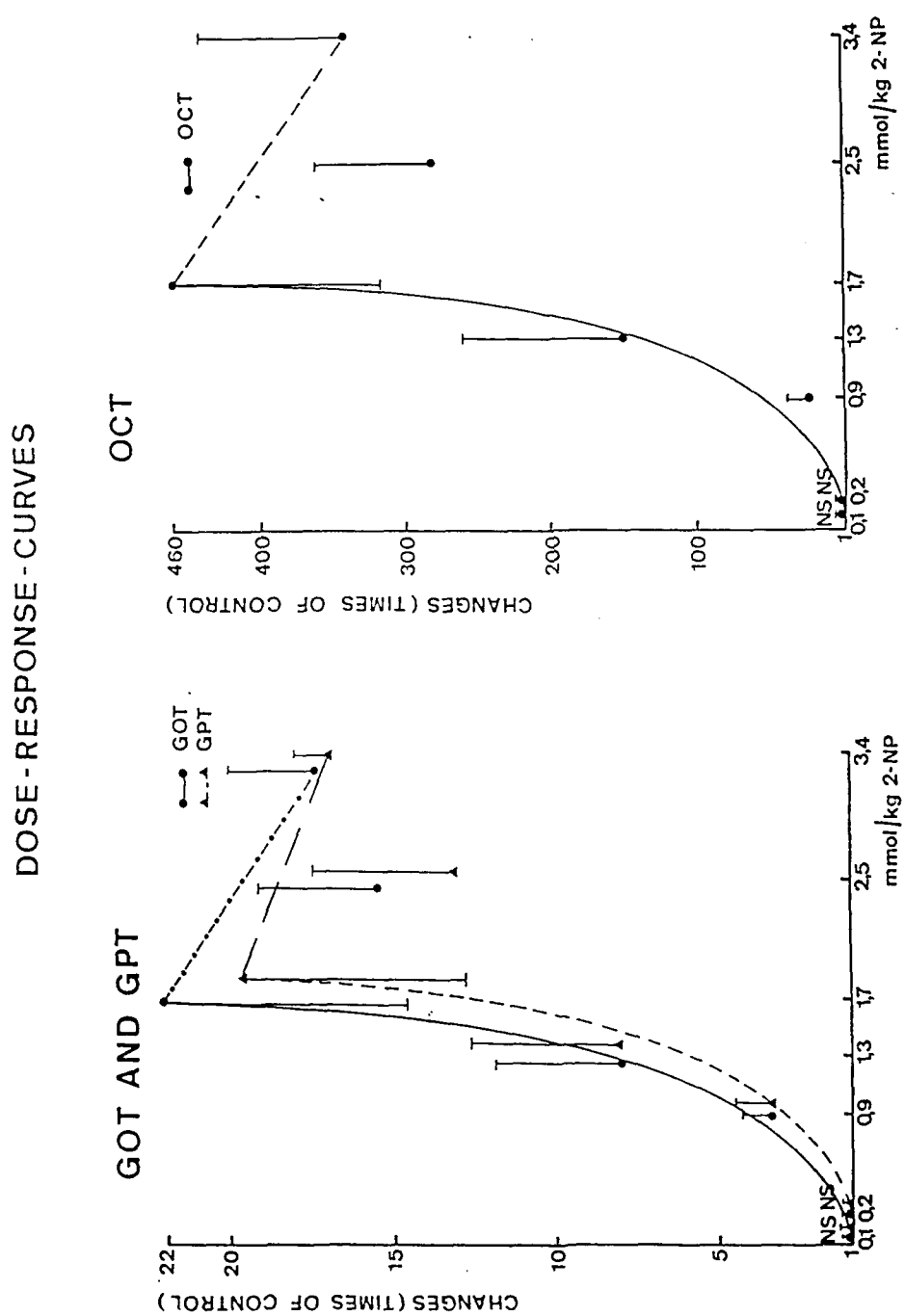


Fig. 15. Activities of GOT, GPT, and OCT in serum of male rats 8 hours after single IP-injections of 2-NP (doses from 0.13 to 3.4 mmol/kg b.wt). Data are given as multiple changes of controls. NS: not significant (n=6)

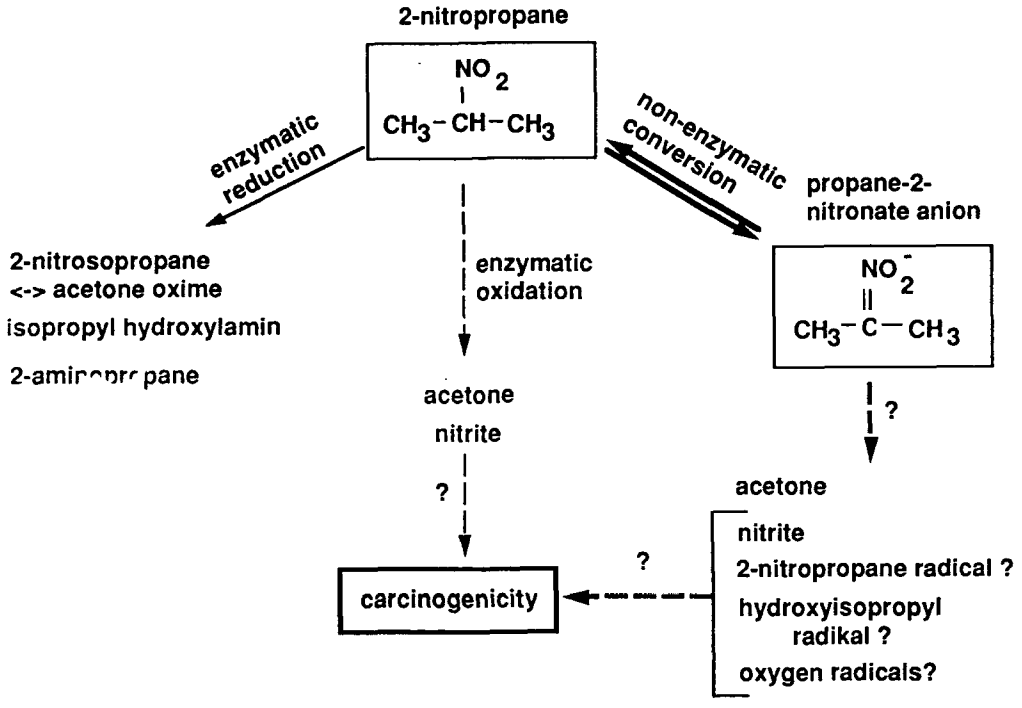


Fig. 16. Metabolic pathways of 2-NP and possible carcinogenic metabolites

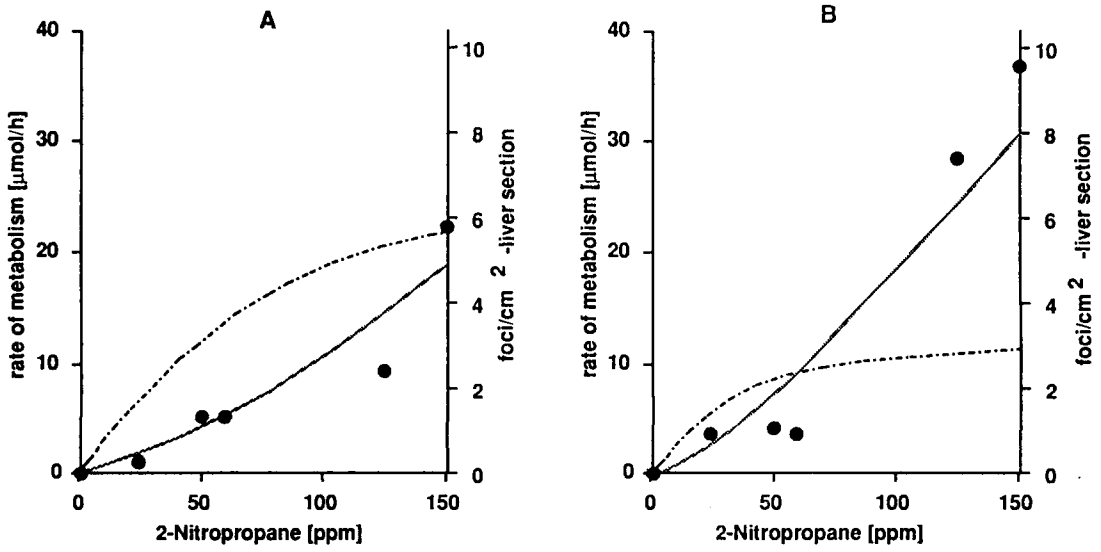


Fig. 17: Correlation of the metabolic rates of the two pathways of 2-NP with its carcinogenic potency in the rat liver foci bioassay
A: female rats B: male rats
Points: numbers of foci incidence after exposure to 2-NP minus the number of foci incidence in the controls
Solid lines: share of the saturable pathway
Dashed lines: share of the non-saturable pathway